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14. ABSTRACT Pre-clinical test results qualify the Cryptosporidium real-time PCR assay as a lead candidate for transition to clinical phase testing. Diagnostic sensitivity results were $\geq 96\%$ to $\leq 100\%$ in testing conducted under laboratory and field conditions. Current commercially available molecular-based diagnostic assay sensitivity is $\geq 95\%$ to $\leq 98\%$ representing the standard that must be met or exceeded to qualify as a candidate for FDA clearance. In addition to test activities, a Cryptosporidium spp Detection Kit pre-IDE document was prepared to serve as a point of departure for discussion with the FDA Office of In Vitro Diagnostic Device Evaluation and Safety (OIVD) on guidance and clarification of specific testing requirements for eventual clearance. During the conduct of RDT&E activities a formal GME training program was established by the investigators. The program provides for scholarly and challenging research opportunities in a real-world environment. Under this project, an Air Force resident physician completed research which directly resulted in advancing Force Health Protection diarrheal disease diagnostic technologies toward						
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Final Report

AF/SGR AFMSA/SG5I RDT&E FY11 - FY13:

PRE-CLINICAL TESTING OF A REAL-TIME PCR ASSAY FOR DIARRHEAL DISEASE
AGENT *CRYPTOSPORIDIUM*

May 16, 2014

Reporting Period: October 1, 2010 to September 30, 2013

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Objectives

1. Pre-clinical trial validated *Cryptosporidium* spp assay qualified for clinical phase testing

Pre-clinical test results qualify the *Cryptosporidium* real-time PCR assay as a lead candidate for transition to clinical phase testing. Diagnostic sensitivity results were $\geq 96\%$ to $\leq 100\%$ in testing conducted under laboratory and field conditions. Current commercially available molecular-based diagnostic assay sensitivity is $\geq 95\%$ to $\leq 98\%$ representing the standard that must be met or exceeded to qualify as a candidate for FDA clearance. Results are provided in final reports.

In addition to test activities, a *Cryptosporidium* spp Detection Kit pre-IDE document was prepared to serve as a point of departure for discussion with the FDA Office of In Vitro Diagnostic Device Evaluation and Safety (OIVD) on guidance and clarification of specific testing requirements for eventual clearance.

2. Report describing ABI 7900 and RAPID/JBAIDS pre-clinical test results

Testing was successful (See Results section). Analytical test results are shown in Appendix C.

3. Completed PEC, NEC, IPC and comparator test evaluations

Controls and comparator test are established (See Results section).

4. Quarterly Progress and Expense Analyses Reports and Final report submitted to the Defense Technical Information Center (DTIC)

Copies of reports can be obtained through Project Manager, 59th MDW/ST.

5. Graduate Medical Education (GME) research project completed

During the conduct of RDT&E activities a formal GME training program was established by the investigators. The program provides for scholarly and challenging research opportunities in a real-world environment. Under this project, an Air Force resident physician completed research which directly resulted in advancing Force Health Protection diarrheal disease diagnostic technologies toward clearance. A research project was completed, abstract prepared, and poster presented at a medical symposium. The resident successfully completed WHAMC Pathology Department Research Elective 144. The project is described in the Results section. Project activities are provided in Appendix A and course description and requirements are provided in Appendix B.

Summary

The objectives of this study were accomplished. Real-time diarrheal disease causative agent detection capability was advanced through pre-clinical test phase. The GME component of this study was successfully completed.

The results of this study support qualification of the assays as candidates for FDA clearance as well as for use in environmental (non-human) surveillance. As such, a pre-investigational device exemption (pre-IDE) document was prepared. The pre-IDE document describes the detection technology and its intended use, proposed analytical testing and clinical evaluation strategies. The intent of FDA guidance meetings are to ensure that proposed testing strategy is in line with current OIVDES thinking and is sufficient to support a pre-market notification application. Investigational device exemption (IDE) will all allow use in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PMA) application or a Premarket Notification [510(k)] submission to FDA. Included in FDA OIVDES guidance meetings will be discussion on potential pre-IDE submissions for high throughput systems (HTS) and microarray systems. The above activities will require additional funding under a separate protocol.

This project was funded by the Air Force Medical Support Agency (AFMSA), Research, Development and Innovations Directorate (SG5I), Office of the Surgeon General (AF/SGR) Falls Church, Virginia and the Military Infectious Diseases Research Program (MIDRP), USAMRC, Fort Detrick, Frederick, Maryland. Project activities were conducted by the Enteric Diseases Department, Armed Forces Research Institute for the Medical Sciences (AFRIMS) and Clinical Research Division (CRD)/59th MDW. This project was jointly funded and executed under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2013. Agreement No.: DODI 4000.19; AFI 25-201).

Products Completed

Point-of-care high throughput system (HTS) and deployable real-time PCR detection capability for diagnosis of etiologic agents of diarrheal disease were accomplished. Pre-clinical test phase demonstrated that the *Cryptosporidium* spp. assay met the objective diagnostic sensitivity. The assays proved specific in testing using a broad panel of clinically significant and genotypically similar organisms.

Purpose

The work completed under this project is follow-on to joint projects completed through previous AF/SGR AFMSA/SG5I funded efforts undertaken by the 59th MDW and Department of Enteric Diseases, AFRIMS. Success in these collaborative efforts has positioned military significant disease agent diagnostics for FDA clearance. Under this project, advanced to clinical test phase is a real-time PCR assays for *Cryptosporidium* spp. The associated training objective provided a scholarly and challenging opportunity in GME.

Pre-clinical testing of a previously established *Cryptosporidium* spp. assay was conducted using two functionally distinct FDA cleared real-time PCR instruments. The first is state of the art laboratory equipment, the Fast Real-Time PCR System (Applied Biosystems, Inc. 7900). This HTS is capable of rapidly screening large numbers of samples, hundreds to thousands, per day. Rapid identification of bacterial disease agents by HTS screening of clinical and environmental samples provides for efficacious treatment and disease prevention. The second PCR instrument is the portable, field-deployable DoD Joint Biological Agent Identification and Diagnostic System (JBAIDS). Disease outbreaks often occur in developing regions and often coincide with natural or man-made disasters. In situations of underdeveloped or failing health care infrastructure, the JBAIDS provides a valuable aid in disease surveillance and diagnosis. The

JBAIDS is deployed in hours and capable of operating independently of conventional laboratory infrastructure. Pre-clinical test results reported here will be used to seek funding for clinical phase testing as well as biosurveillance kit development.

Problem

Cryptosporidiosis is a waterborne diarrheal disease caused by parasites of the genus *Cryptosporidium* that is recognized as one of the most common causes of disease in humans. Cryptosporidiosis is endemic throughout the world, including every region of the United States. The DoD rates diarrheal disease as the number 1 infectious disease that threatens deployed military forces and as such the Joint Program Executive Office (JPEO) has designated *Cryptosporidium* as a threat agent (Block 1, Tier 2) to deployed military forces.

Results of Pre-clinical Performance

Diagnostic Sensitivity - pre-clinical phase test results show that the JBAIDS TaqMan *Cryptosporidium* spp. assay met the objective diagnostic sensitivity, $\geq 95\%$ to $\leq 98\%$ (Table 1). Specificity test results reported here, and in previous testing, showed that the assays are specific (Appendix C).

Table 1. Diagnostic sensitivity of *Cryptosporidium* PCR assay direct detection from stool

Assay	RAPID Singleplex AFRIMS, Sept 2013 Sensitivity (%)	RAPID Field Test Singleplex Nepal, Mar 2009 Sensitivity (%)	Acid-Fast Staining <i>Cryptosporidium</i> AFRIMS Sensitivity (%)
<i>Cryptosporidium</i> spp	96% (25/26)	100% 34/34	79% 27/34

Cut-off Ct 42

This study was conducted to determine the performance of the assays for relevant specimen types claimed in future labeling. The study protocol provides patient sample inclusion and exclusion criteria, type and number of specimens, directions for use, and statistical analysis information will be used for potential premarket submission. The specimen types (strains) and total number of samples were based on diarrheal disease epidemiological data. The objective number of samples was a minimum of 30 confirmed positive using the reference method. Pre-clinical studies were conducted at a single facility utilizing a single laboratory. Testing was conducted by experienced and trained personnel at the Department of Enteric Diseases, AFRIMS. This laboratory is the only DoD facility currently conducting diarrheal disease agent clearance activities. Test activities were conducted following GLP guidelines. The study population included archived nucleic acid extracts from individuals who presented with diarrheal disease. Results were compared using the established reference method (culture). In addition to culture sequencing of amplicon was used for confirmation testing.

Interference Study

Stool presents a relatively complex challenge in sample preparation. Stool harbors an array of PCR interfering substances that must be removed during the nucleic acid extraction process to help assure an efficacious level of diagnostic sensitivity.

A preliminary interference study was successfully completed using clinically relevant conditions. The interferent used was human blood which represents the primary PCR inhibitory substance encountered in stool specimens. The interferent was tested at the potentially “the worst case” concentration (10% w/w) using *Cryptosporidium parvum* from stool to assess the potentially inhibitory effects. Interference testing was conducted at LoD and 1000X LoD concentrations of organism to assess inhibitory effects as well as to assess potential for cross-contamination.

There was no significant difference in *Cryptosporidium* spp. assay Ct values for stool prepared with spiked-blood and non-spiked samples (below). Throughout testing there was no indication of cross-contamination. Study design and JBAIDS screen shots are shown below.

Target	Undiluted	LOD		1,000 LOD	
		Oocysts	~Ct	Oocysts	~Ct
CR	1.0×10^6 oocysts	10 oocysts	35	10^4 oocysts	25

L1: Lysis

L2 : Added Whole blood in Lysis (40uL:1.4mL)

T10 : 10^3 LOD of CR stool + L1 (60 mg : 1.4mL)

T11 : 10^3 LOD of CR stool + L2 (60 mg : 1.4mL)

T12 : LOD of CR stool + L1 (60 mg : 1.4mL)

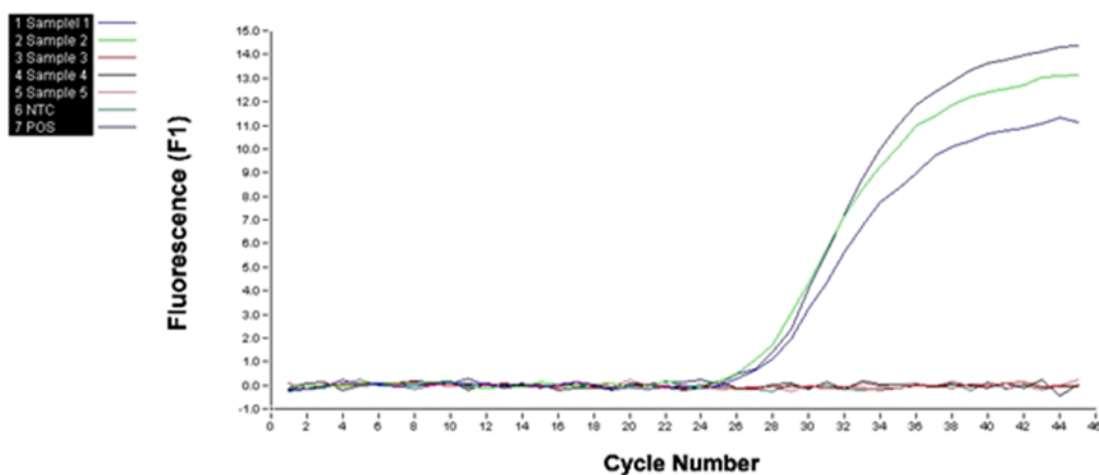
T13 : LOD of CR stool + L2 (60 mg : 1.4mL)

T14 : Negative stool + L1 (60 mg : 1.4mL)

Extraction by Qiagen stool kit



Elute 60 uL



Name	Tube	ID	Ct
Samp1	T1	10^3 LOD-CR	27.35
Samp2	T2	10^3 LOD-CR + Inhibitor	36.90
Samp3	T3	LOD-CR	-
Samp4	T4	LOD-CR + Inhibitor	-
Samp5	T7	Neg Stool	-
Samp6	N	NTC	-
Samp7	P	POS	27.62

Positive Extraction Control (PEC), Negative Extraction Control (NEC), Internal Positive Control (IPC) and Comparator Test

Negative Controls

No template control (NTC) - The NTC reaction contains buffer and all of the assay components except nucleic acid. These controls ruled out contamination with target nucleic acid or increased background in the amplification reaction. No template control reactions were manufactured by Idaho Technology, Inc (now BioFire Diagnostics), Salt Lake City, Utah.

Negative sample control

The negative sample control contained non-target nucleic acid. When used to evaluate extraction procedures it contained whole organism to reveal non-specific priming or detection and to indicate that signals were obtained in the absence of target sequences. Negative sample control materials included:

- Patient specimen from an infected individual
- Samples containing a non-target organism

Positive Controls

Positive control for complete assay - The positive control contained well characterized target nucleic acid to control the entire assay process, including DNA extraction, amplification, and detection. It was designed to mimic a patient specimen and to be run as a separate assay, concurrently with patient specimens at a statistically significant frequency. As stated in the proposal, the development and validation of a positive control will require additional funding through follow-on proposal.

Positive control for amplification/detection (PTC) - The PTC for amplification/detection contained purified well characterized target nucleic acid. The PTC was designed to report fluorescence at or near the LoD. The PTC controlled the integrity of the patient sample and the reaction components when negative results were obtained and to indicate that the target is detected if present in the sample. The PTC was manufactured by Idaho Technology, Inc (now BioFire Diagnostics), Salt Lake City, Utah.

Internal Positive Control - Various candidate PCR internal positive controls (IPC) were evaluated for down-selection and testing. The IPC is a non-target nucleic acid sequence that is co-extracted and co-amplified with the target nucleic acid. It will control the integrity of the reagents (polymerase, primers, etc.), PCR instrument function, and the presence of inhibitors in the samples. The human housekeeping gene β -actin was selected for IPC development. As stated in the proposal, the IPC will require additional funding through follow-on proposal.

Comparator Test – gold standard methodology was used as the comparator test (culture) with confirmation testing of amplicon by DNA sequencing. In addition, diagnostic sensitivity test results were compared to *Cryptosporidium* acid-fast staining.

Assay Storage Conditions - A thermal stability study demonstrated that the assays generate equivalent results at several time points throughout the duration of the recommended storage and at both ends of the recommended temperature range. Thermal stability studies were conducted under the GME project. See Results section “Graduate Medical Education Project”.

ABI 7900 Transfer

Transfer of JBAIDS formatted assays to the ABI 7900 was successfully completed. Study design is shown in Table 2. Optimized assay formulations and reaction conditions are shown in Table 3. Average Ct Values of Standard Curves from triplicate of 4-folds serial dilutions of ETEC, Shigella, and CR assays are shown in Table 4. Standard curves are shown in Figures 1 - 5. Limit of detection estimation derived from standard curve are shown in Table 5. These data include results from both 'JBAIDS ETEC/Shigella' and 'JBAIDS Cryptosporidium' projects.

Standard cuve for estimate LOD

Materials and Methods:

1. Isolated colonies for each pathogens were selected and picked from sub culture agar plate (1 loopful)
2. The colonies were suspended in normal saline separately.
3. Suspended colonies were measured at 625 nm and adjusted to 0.5 McFarland (OD_{625nm} 0.088 – 0.133).
4. Nucleic acids extraction was performed using boiling method.

Table 2. OD_{625nm} and Nanodrop measurement of extracted DNA

Strain	OD_{625nm}	Concentration of cell suspension (cell/mL)- 0.5 McFarland
ETEC-STIa	0.106	1.5×10^8
ETEC-STIb	0.091	1.5×10^8
ETEC-LT	0.090	1.5×10^8
Shigella-ipaH	0.088	1.5×10^8
Isolates of <i>Cryptosporidium parvum</i> from Waterborne Inc., using Qiagen extraction kit		
Parasite	NanoDrop measurement	Concentration of undiluted DNA sample
Cryptosporidium- CR	25.5 $\eta g/\mu L$	10^6 oocysts/ μL

PCR amplification and detection

Table 3. The PCR reactions contain the following reagents at specified concentrations:

Reagents	ETEC (STIa, STIb, LT)	Shigella (ipaH)	Cryptosporidium (18S r-RNA)
10X bufferA		1X	
dNTPs (mM)		0.2	
TaqGlod		0.5 U	
Mg ²⁺ (mM)	2.5	2.0	3.0
Probe (nM)	100	100	100
Primer (μM)	0.2	0.2	0.2
Template (μL)		2	
Total Vol. (μL)		20	

Thermo cycler Condition: 95 °C 10 min, 40Cyclers of 95 °C 15 sec and 60 °C 1 min. Template was diluted serially at 4 folds from dilution 1 to dilution 9 (approximately 3.75×10^7 - 5.72×10^2)

Table 4. Average Ct Values of Standard Curves from triplicate of 4-folds serial dilutions of ETEC, Shigella, and CR assays

Strain	ETEC-STIa assay		ETEC-STIb assay		ETEC-LT assay		ipaH assay		CR assay	
Dilution	Ct Av.	STDEV	Ct Av.	STDEV	Ct Av.	STDEV	Ct Av.	STDEV	Ct Av.	STDEV
D1	21.48	0.431	21.43	0.333	22.34	0.086	19.48	0.154	20.12	0.294
D2	24.58	0.194	23.63	0.332	24.29	0.362	21.29	0.241	22.02	0.244
D3	26.75	0.460	25.69	0.159	26.66	0.220	23.51	0.122	23.77	0.179
D4	28.94	0.448	27.80	0.108	28.75	0.119	25.47	0.196	25.80	0.208
D5	30.71	0.226	29.85	0.150	30.80	0.106	27.58	0.304	28.23	0.212
D6	33.16	0.342	32.35	0.400	32.98	0.393	29.54	0.169	29.98	0.346
D7	35.47	0.216	34.65	0.242	34.92	0.289	31.38	0.100	31.96	0.513
D8	37.54	0.478	37.44	1.560	36.87	1.064	33.71	0.189	34.48	0.616
D9	NA	NA	NA	NA	NA	NA	35.77	1.385	36.76	0.940
Threshold	0.04		0.04		0.04		0.1		0.1	
R ²	0.995		0.989		0.993		0.996		0.997	
Y-Intercept	50.934		52.822		49.455		47.393		39.303	
Slope	-3.699		-3.776		-3.489		-3.486		-3.395	
Figure	1		2		3		4		5	

Av. = Average; STDEV = Standard deviation

Figure 1. Standard curve for ETEC-STIa

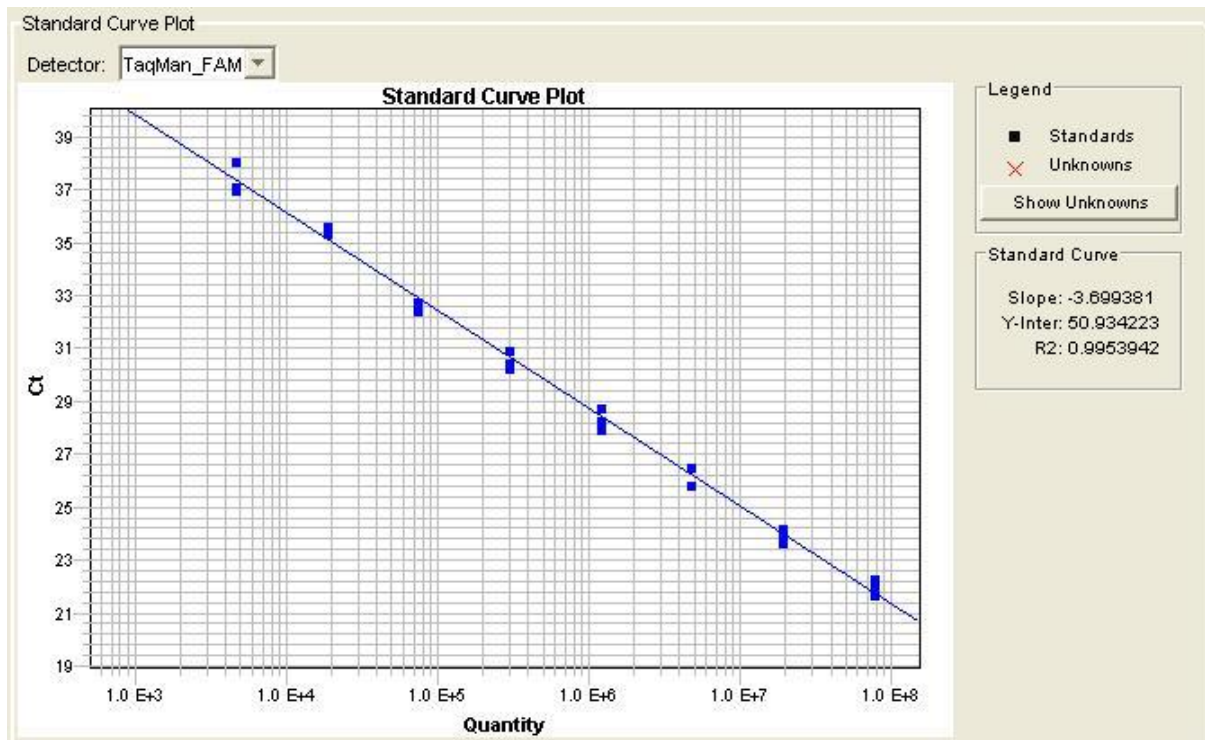


Figure 2. Standard curve for ETEC-ST1b

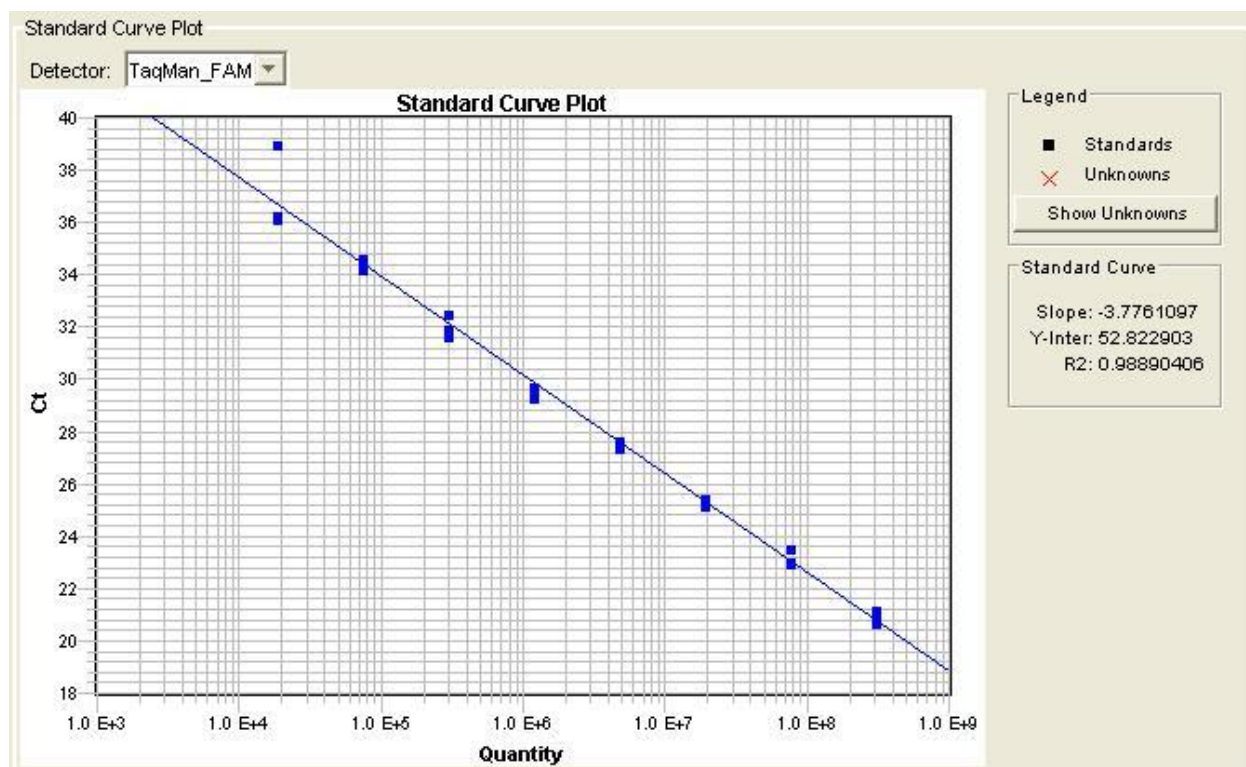


Figure 3. Standard curve for ETEC-LT

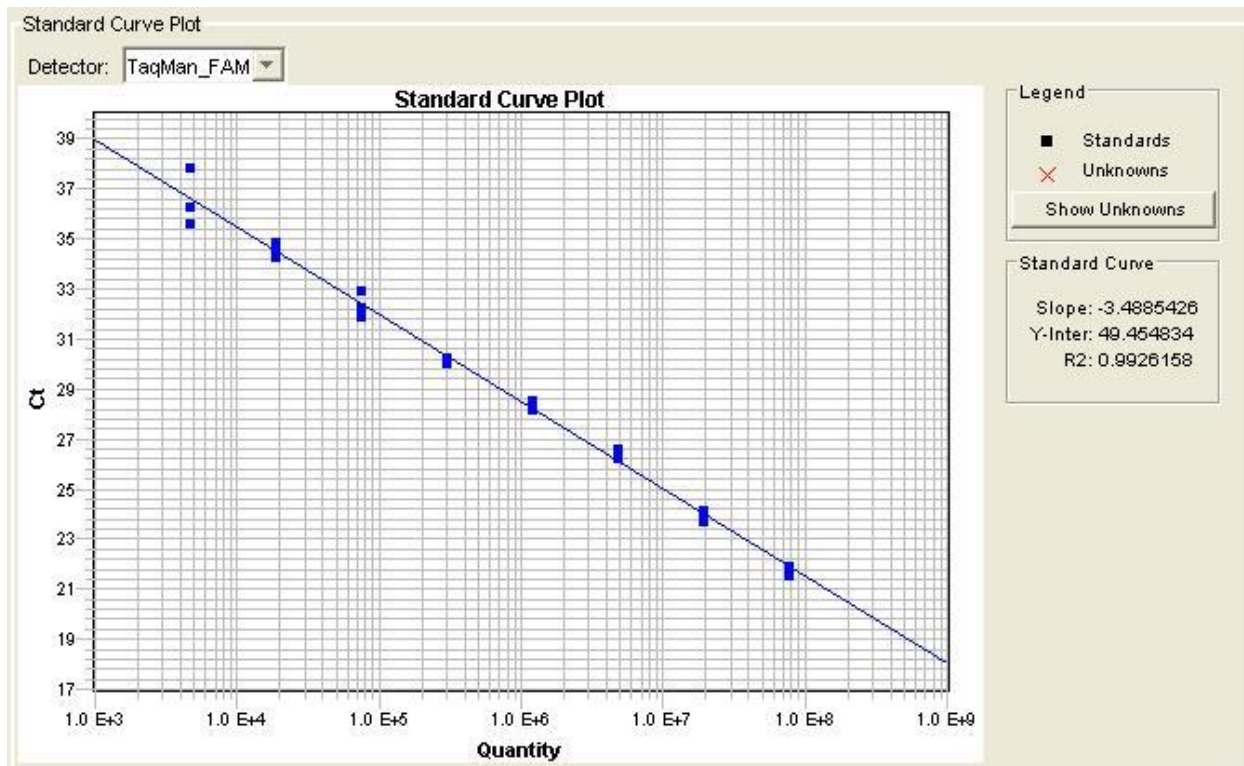


Figure 4. Standard curve for *Shigella-ipaH*

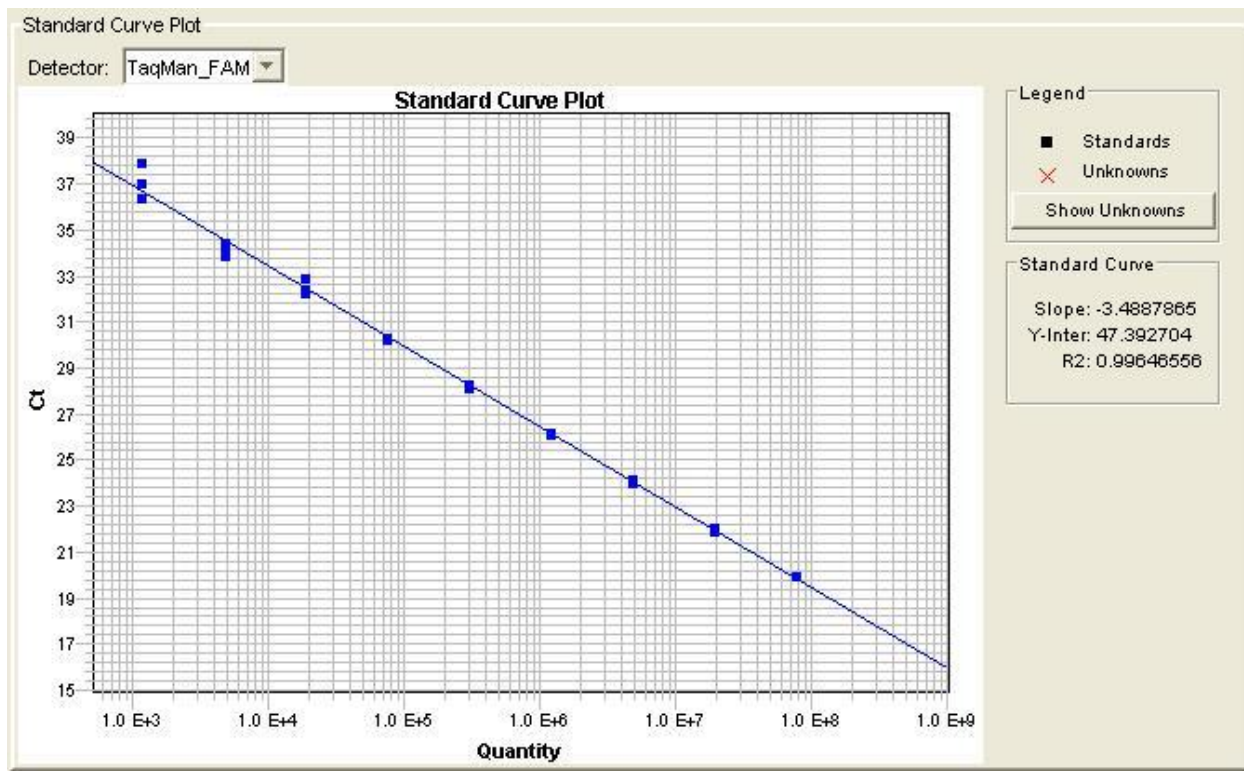


Figure 5. Standard curve for *Cryptosporidium*

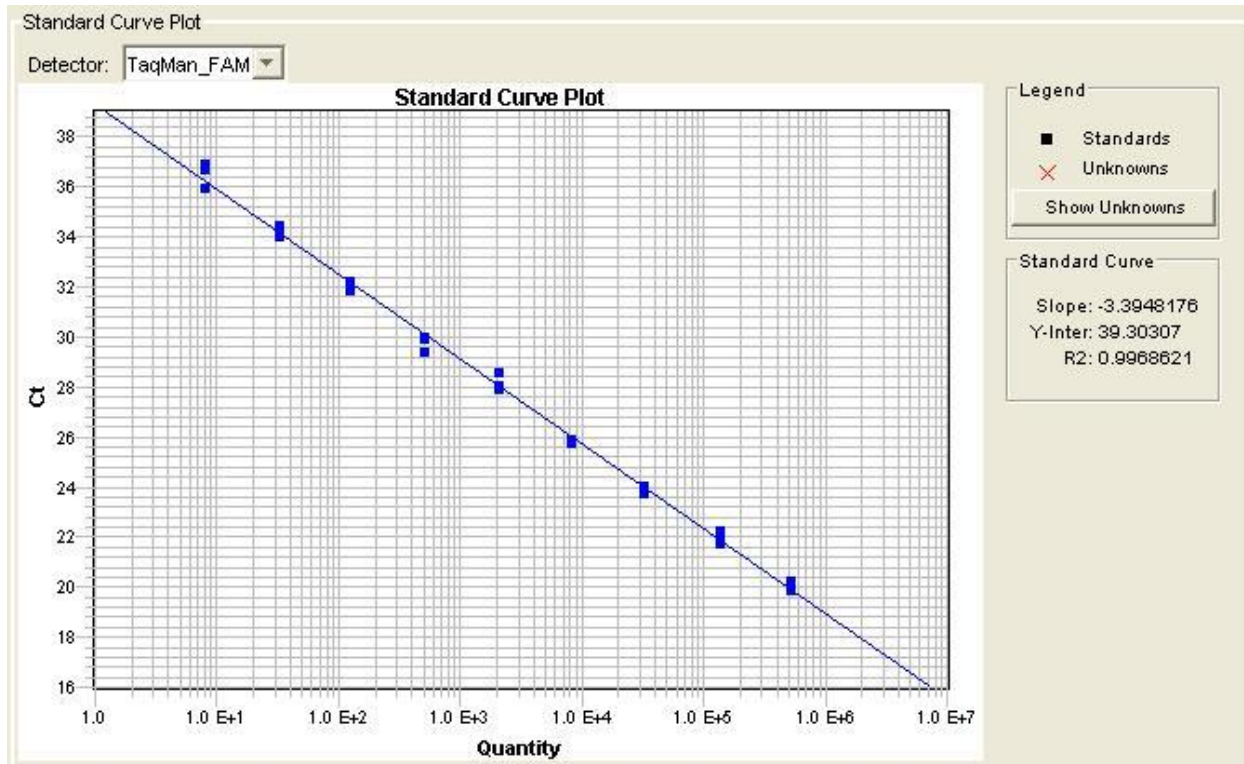


Table 5. LOD estimation derived from standard curve

Assay	Dilution (calculated concentration)*	ABI7900		LOD of RAPID CFU/mL
		Ct average	STDEV**	
ETEC-STIa	D8 (2.29×10^3 CFU/mL)	37.54	0.478	1.5×10^4
ETEC-STIb	D8 (2.29×10^3 CFU/mL)	37.44	1.560	1.5×10^4
ETEC-LT	D8 (2.29×10^3 CFU/mL)	36.87	1.064	1.5×10^4
Shigella-ipaH	D9 (5.72×10^2 CFU)	35.77	1.385	1.5×10^3
Cryptosporidium	D9 (3.81 Oocysts/ μ L)	36.76	0.940	1×10^1

*The last detectable dilution was used to calculate the CFU/mL from starting concentration of 1.5×10^8 CFU/mL. However, in a LOD experiment, the dilution used for this calculation should be the dilution that shows consistent detectable Ct throughout the experiment which does not necessarily have to be the last detectable dilution.

**STDEV = Standard deviation

Graduate Medical Education Project

Graduate Medical Education training was conducted during 1 - 31 May, 2012 at the U.S. Army Medical Component, Armed Forces Institute of Research of Medical Sciences (AFRIMS), Bangkok, Thailand.

GME Resident: Maj Robert O. Brady, M.D.

GME Mentor: Col Joseph Peter Ray Pelletier, M.D.

Principal Investigator: James C. McAvin and Co-PI: COL Carl Mason, Chief, Department of Enteric Diseases, AFRIMS.

Project results were formatted as an abstract and presented at a medical symposium; The American Society of Clinical Pathologists Symposium, Boston, MA, 31 OCT - 3 NOV, 2012 (poster presentation).

Continued Polymerase Chain Reaction Assay Stability Over Time and In a Changing Thermal Environment

Robert O. Brady MD, James C. McAvin, J Peter R Pelletier MD
Wilford Hall Ambulatory Surgical Center/59 MDW

Surveillance of bacterial diarrheal agents is at the forefront of world health disease prevention. Systems that utilize environmentally stable assay reagents help decrease response times, reduce costs, and save lives during times of suspected outbreaks. We performed real-time polymerase chain reaction assay stability tests on freeze-dried *Shigella* ipaH, enterotoxigenic *E. coli* (ETEC) ST1b, ETEC LT, ETEC ST1a, *Cryptosporidium*, and *Leptospira* (Idaho Technologies, Salt Lake City, Utah) in variety of storage environments (-20°C approximately 3 years, 4°C 14 days, and 16°C to \geq 40°C 14 days). Initial assay stability testing was conducted after reagent production in 2009 and then stored at -20°C until 2011 when favorable stability testing was conducted. We tested assays remaining at -20°C with the same protocols and instrument, using the above storage parameters; and compared data with original testing results. Testing showed overall assay stability after storage at -20°C (3 years), at 4°C, and at varied heat/humidity environments (16°C to \geq 40°C, 50-100% humidity) for two weeks. *Shigella* ipaH (SD range 0.15-0.45, 95% CI), ETEC LT (SD range 0.042-1.24, 95% CI), ETEC ST1b (SD range 0.063-0.79, 95% CI), and *Cryptosporidium* (SD range 0.057-2.11, 95% CI) all appear stable in tested environments. *Leptospira* showed long-term stability at -20°C (SD range 0.099-1.51, 95% CI). ETEC ST1a shows possible degradation at -20°C, since original testing (SD range 0.099-2.63, 95% CI). In conclusion, these new data show the potential for prolonged storage (-20°C) without significant degradation of assay performance. ETEC ST1a needs further evaluation for degradation at -20°C. After three years at -20°C, assays stored at 4°C for two weeks show no degradation in performance. Lastly, after three years at -20°C we showed that these assays are likely stable for two weeks when stored in an ambient tropical environment. These results give users in austere environments possible flexibility with storage conditions--reducing costs and logistic burdens.

Continued Polymerase Chain Reaction Assay Stability Over Time and In a Changing Thermal Environment

Robert O. Brady M.D., J Peter R Pelletier M.D., James C. McAvin
Wilford Hall Ambulatory Surgical Center, San Antonio TX

Introduction

Surveillance of bacterial diarrheal agents is at the forefront of world health disease prevention. Systems that utilize environmentally stable assay reagents help decrease response times, reduce costs, and save lives during times of suspected outbreaks.



Figure 1: RAPID System in the lab.
(Ruggedized Advanced Pathogen Identification Device)

Methods and Materials

We performed real-time polymerase chain reaction assay stability tests on freeze-dried *Shigella* ipaH, enterotoxigenic *E. coli* (ETEC) ST1b, ETEC LT, ETEC ST1a, *Cryptosporidium*, and *Leptospira* (Idaho Technologies, Salt Lake City, Utah) in variety of storage environments (-20°C approximately 3 years, 4°C 14 days, and 16°C to ≥40°C 14 days). Initial assay stability testing was conducted after reagent production in 2009 and then stored at -20°C until 2011 when favorable stability testing was conducted. We tested assays remaining at -20°C with the same protocols and instrument, using the above storage parameters; and compared data with original testing results.

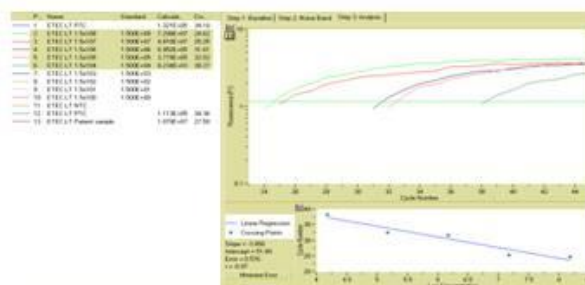


Figure 2: ETEC LT Initial Reagent Stability Test (Time = 0 weeks)

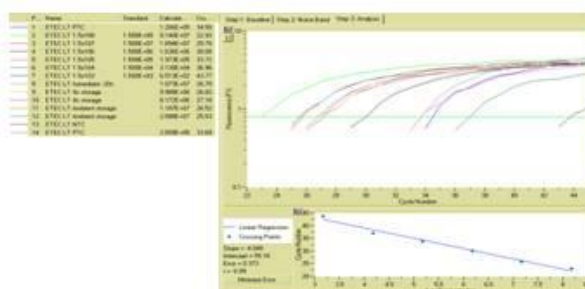
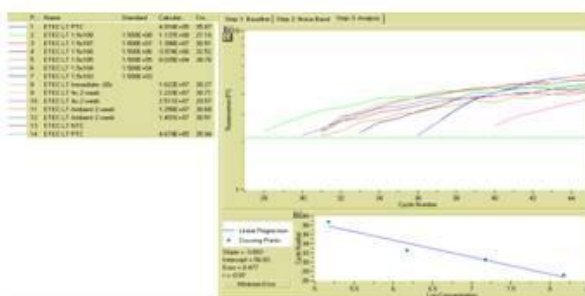


Figure 3: ETEC LT Reagent Stability Test after exposure to changing the thermal environments (Time = 1 week)



Conclusion

Work conducted under this study advanced real-time diarrheal disease causative agent diagnostic assays through pre-clinical test phase. Results reported qualify the assays as lead candidates for clinical phase testing. A GME training program was established which provided for scholarly and challenging research opportunity in a real-world environment.

Appendix A

Graduate Medical Education

I. Project Activities Completed (GME)

The investigators conducted the following GME activities: preparation of course materials, development of a research project, preparation of a Research Plan and Training Schedule, integration (and de-integration) of the GME laboratory, reagent and sample preparation, coordination and execution of GME research activities, mentored the resident in proposal writing and results reporting, maintained daily log of resident activities and progress, assured the safety and wellbeing of GME participants. The GME Research Plan and Training Schedule and detailed description of activities are provided below.

A. Demonstrated knowledge and skill in;

- I. Initiative development and project definition.
- II. Request For Proposals (RFP) - announcement search.
- III. RFP - application process.
- IV. Proposal development - statement of work (SOW).

Exercise 1: Essay on the Scientific Method (~ 250 words): COMPLETED.

Exercise 2: Define a project, locate an appropriate funding source, and write a pre-proposal/SOW: COMPLETED.

Pre-proposal/SOW: "Leptospirosis GME" (Exercise 2)

B. Demonstrated knowledge and skill in;

- I. Role and function of Institutional Review Board (IRB)
- II. IRB protocol requirements and associated documentation.
- III. IRB review process

Exercise 3: Complete CITI and HIPPA compliance training: COMPLETED.

Exercise 4: Essay on IRB function and purpose of informed consent (~ 250 words): COMPLETED.

C. Demonstrated ability to;

- I. Independently conduct a sub-component of an ongoing research project.
- II. Prepare a scholarly research abstract.
- III. Successfully submit and present results at a scientific meeting or symposium.

Exercise 5. Complete a laboratory practical on sample preparation and analyses: COMPLETED (100% concordance using a 10 sample test panel).

Exercise 6. Complete statistical analyses of results: COMPLETED.

Exercise 7: Write a research abstract: COMPLETED.

An abstract was written and the associated poster presentation material prepared.

“Continued TaqMan Polymerase Chain Reaction Assay Stability Over Time and In a Changing Thermal Environment”

Exercise 8: Abstract submitted on 30 MAY 12; American Society of Clinical Pathologists meeting Boston, MA 31 OCT - 3 NOV, 2012: COMPLETED.

Presentation of results pending acceptance for poster presentation: TO BE COMPLETED.

Course Summary and Schedule

Research Elective 144

7 May to 1 June, 2012

Goals and Objectives: to gain a better understanding of the scientific method and the acquisition of new knowledge through a mentored research experience. The resident will demonstrate ACGME competencies in medical knowledge, practice-based learning and improvement, interpersonal and communication skills, and professionalism.

General objectives are to:

1. Acquaint the resident with a particular area of medical-related research.
2. Teach the resident appropriate research techniques and research design.
3. Assist the resident to complete and write up for publication the results of their research

Specific resident learning objectives for the research project are:

To learn to develop a research question (Medical Knowledge and Practice-Based Learning).

To learn to access, critique, and assimilate the current medical literature pertaining to the research topic (Practice-Based Learning).

To gain an understanding of the scientific method by learning to write an IRB approved research protocol (Practice-Based Learning).

To learn and understand the purpose of informed consent and the regulatory approval process in the setting of research ethics by completing the HIPPA compliance training and obtaining IRB approval for the proposed research project (Professionalism and Systems-Based Practice).

To perform the research and develop the necessary skills required to do this such as laboratory techniques and computer skills (Practice-Based Learning).

To learn and apply the appropriate data analysis and basic biostatistics needed for the project (Practice-Based Learning).

Outcomes assessment: Subjective - A standard competency-based trainee evaluation will be completed at the end of the rotation by the faculty research mentor.

Evaluation

All projects are graded by the Program Director or Associate Program Director using the standard score sheet on the SAUSHEC web site (see appendices A and B). A minimum score of 60 is required to graduate. It is highly recommended that the resident strive for first authorship on a publication in any of the categories listed in the appendix.

Course objectives: the objective of this course is to meet Resident Program requirements in the conduct and completion of a research rotation.

The resident will demonstrate knowledge and proficiency in:

1. IRB protocol and associated documentation preparation and progress reporting.
2. Proposal preparation, funding application preparation and submission, and reporting process.
3. Operation of DoD approved analytic instrumentation (RAPID/JBAIDS) and conduct testing under deployed conditions.
4. The conduct and completion of a research project and results reporting.
5. The preparation of a scholarly abstract and submission to a scientific meeting or symposium.
6. Presentation of results at a scientific symposium, conference, or meeting.

The student will meet or exceed the requirements for completion of Research Elective 144. At the conclusion of the course the student will have prepared a research pre-proposal and statement of work that is suitable for submission for funding. The student will be prepared to submit the associated IRB documentation. The student will have demonstrated the ability to independently conduct and complete a research project and report the results.

Week 1

7 May, 2012

Course Preparation and Travel to Field Site

Monday 7

Literature review and conduct literature search (suggested key words; diarrheal disease, ETEC, *Shigella*, *Cryptosporidia* diagnostics, real-time PCR, RAPID/JBAIDS).

Send itinerary and contact information to PI.

Tuesday 8

Review and organize travel file (travel documents, readiness file, GME Training Plan, ETEC/*Shigella* and *Cryptosporidium* proposals, and research articles).

Wednesday 9

Pack and prepare for departure. Confirm link-up time/location with travel companions and confirm with PI contact information, arrival time and pick-up location.

~ **08.00** - Depart US for AFRIMS, Bangkok

Thursday 10

23.00 - Arrive Bangkok, transportation to lodging (PI will meet at BKK Airport)

24.00 - Hotel (Royal View Bangkok)

Friday 11 - Orientation

Course overview, RAPID orientation, and lab orientation; badge acquisition.

Week 2

14 May, 2012

Orientation and Training

Monday 14

0930- 1130 - Course Goals & Objectives, Project Background, and Technology Overview (EDS Dept. Conf. Room, AFRIMS) – established associated course exercises.

AM:

- Brainstorm session of additional abstract ideas.
- Allocate assays for new thermal stability study (in addition to longevity study) to store at 4c and RT (in backpack).
- Reviewed molecular biology presentation and associated pre-experimental documents.
- Initiated documentation for project, notebook, and data collection.

PM:

- Familiarization session with RAPIDS machine and performed test run of RAPIDS using Shigella assay positive control.
- Reviewed results and defined terms associated with results/analysis performed by the RAPIDS computer program.

GME Goals and Objectives

The student will meet or exceed the requirements for completion of Research Elective 144. At the conclusion of the course the student will have prepared a research pre-proposal and statement of work that is suitable for submission for funding. The student will be prepared to submit the associated IRB documentation. The student will have demonstrated the ability to independently conduct and complete a research project and report the results.

A. Demonstrated knowledge and skill in;

- I. Initiative development and project definition.
- II. Request For Proposals (RFP) - announcement search.
- III. RFP - application process.
- IV. Proposal development - statement of work (SOW).

Exercise 1: Essay on the Scientific Method (~ 250 words):

Exercise 2: Define a project, locate an appropriate funding source, and write a pre-proposal/SOW:

B. Demonstrated knowledge and skill in;

- I. Role and function of Institutional Review Board (IRB)

- II. IRB protocol requirements and associated documentation.
- III. IRB review process

Exercise 3: Complete CITI and HIPPA compliance training: COMPLETED.

Exercise 4: Essay on IRB function and purpose of informed consent (~ 250 words):

C. Demonstrated ability to;

- I. Independently conduct a sub-component of an ongoing research project.
- II. Prepare a scholarly research abstract.
- III. Successfully submit and present results at a scientific meeting or symposium.

Exercise 5. Complete a laboratory practical on sample preparation and analyses:

Exercise 6. Complete statistical analyses of results:

Exercise 7: Write a research abstract:

Exercise 8: Submit abstract and present results:

1230-1630 - review of course study materials.

Tuesday 15

0930- 1130 - Overview of Proposal Submission Process: Scientific Method, Proposal Application Process, IRB Submission Process (EDS Dept. Conf. Room, AFRIMS).

1230-1630 – RAPID check-out and review of course study materials.

- Continued familiarization of RAPIDS machine and software.
- Pipetting exercise on multiple volumes and methods to decrease errors.
- Performed Shigella IHAP dilutions and ran standardized curve on RAPIDS machine.
- Reviewed results of Shigella standardized curve and planned out tests on ETEC ST1b, LT, 1a, and Crypto.

Wednesday 16

0900-1200 - Proposal Development, IRB Review Process, Molecular Biology Tutorial (EDS Dept. Conf. Room, AFRIMS).

1300-1700 - Proposal Development: proposal topic and documentation preparation (pre-proposal). Review of Molecular Biology Tutorial study materials.

- Performed ETEC ST1b and ETEC LT standard titration curves.

- Reviewed data of above tests and planned ETEC 1a and Crypto tests.
- Molecular biology review session.

Thursday 17

0900-1200 Laboratory Tour and Orientation (Enteric Diseases Dept, AFRIMS)

1300-1700 GME laboratory set up, RAPID system configuration (*Lecture & Training*), **RAPID system check-out** (*Lecture & Training* - RAPID test run conducted with positive controls reactions).

- Performed ETEC ST1a and Crypto titration curves and reviewed test results
- Brainstorm of proposal ideas for future studies.
- Updated RAPIDS test run worksheets from stability curves performed up to now.
- Planned for 1 week stability test of all assays over the coming week.

Friday 18

0800-1130 Qiagen kit extraction (*Lecture & Training*) - stool extraction protocol and PEC development activities conducted.

1130-1700 Results review, research activity briefing, protocol reviews, and proposal development exercise.

- Reading/research review/literature review day

Sat - Sun - Results review, research activity briefing, protocol reviews, and proposal development exercise.

Week 3

21 May, 2012

Training

Monday 21

0800-1200 Sample preparation (*Lecture & Training*) - deployable stool nucleic acid extraction protocol and PEC development activities conducted.

1300-1700 Sample preparation – The student was trained and successfully conducted a limit of detection (LOD) experiment and LOD reproducibility testing using the RAPID freeze-dried assay and relevant extracts.

- Performed 1 week assay stability test for ETEC ST1b, LT, and Shigella; on those kept at 4c and those kept at ambient temperature (backpack).
- Reviewed results and planned for testing on ETEC ST1a, Crypto and Lepto PTC.

Tuesday 22

0800-1200 Nucleic acid preparation - the student was trained and successfully conducted activities toward the development of a deployable stool extraction protocol using the RAPID freeze-dried assay and relevant samples.

1300-1700 Real time PCR detection - the student conducted PCR analyses of stool extract using the RAPID freeze-dried assay and relevant samples. Planned and coordinated follow-on development activities for the deployable stool extraction protocol. Continued work on exercises 1, 2, and 4.

- Performed 1 week assay stability test for ETEC ST 1a, Crypto and Lepto.
- Reviewed results and went over favorable data showing stability at the 1 week mark for the tested reagents (both 4c and ambient temperatures).
- Filled in RAPIDS data worksheets for the previously run assays (21-22 May)
- Completed IRB and SM essays.
- Discussed proposal of a Leptospirosis study dealing with rodent capture, water testing, and blood/urine samples of patients—Further discussion for pre-proposal is needed.
- Work on abstract of continued stability testing at 4c and ambient temperatures (7 and 14 days) of diarrheal agents ETEC and Shigella (Crypto included).

Wednesday 23 0800-1700 (1 hour lunch)

Nucleic acid preparation and real time PCR detection - the student conducted an experiment to determine the LOD of a PCR assay using spiked stool samples at 1.5×10^8 to 1.5×10^0 cfu/ml concentrations. No fluorescence was reported. The student conducted trouble-shooting and learned that the experiment was inadvertently set-up using template. The PCR was repeated using 1.5×10^8 cfu/ml concentration and fluorescence reported at the expected Ct. Continued work on exercises 1, 2, and 4.

- Nucleic acid preparation and real time PCR detection - the student conducted an experiment to determine the LOD of a PCR assay using spiked stool samples and QIAGEN viral RNA kit.

Thursday 24 0800-1700 (1 hour lunch)

Nucleic acid preparation and real time PCR detection - the student conducted an experiment to determine the LOD of a PCR assay using spiked stool samples at 1.5×10^8 to 1.5×10^0 cfu/ml concentrations. Continued work on exercises 1, 2, and 4.

- Nucleic acid preparation and real time PCR detection assay using spiked stool samples and QIAGEN viral RNA kit on ETEC ST1b. Ran test without aid as a practical exercise. Test results were compatible with a positive run from the spiked stool using the RNA kit-exercise practical successful.

Friday 25 0800-1700 (1 hour lunch)

Results review, follow-on research activity planning and coordination, protocol review and revision, and proposal development exercise. Exercises 1 and 4 completed; essays on the “Scientific Method” and “IRB Function and Purpose of Informed Consent.

- Performed first portion of 2 week tests on 4c and ambient temperatures (due to Federal Holiday on Monday), tests on final four samples (both temps) will be completed on Tuesday.

Week 4

28 May, 2012

Training and Data Collection

Results Review, Data Archiving, and Re-deployment

Monday 28 0800-1700 (1 hour lunch)

Real time PCR detection (Proficiency Evaluation) - preparation of a 30 sample test panel consisting of well characterized nucleic acid extracts from archived patient samples.

Continued working on abstract, essays, and paper outlining methods of experiment.

Tuesday 29 0800-1700 (1 hour lunch)

Sample Preparation and Real time PCR detection (Proficiency Evaluation) - the student prepared spiked stool samples using isolates.

- Performed final two runs with the last four samples (1b and Shigella), both runs successful.
- Compiled data charts and continued to organize lab notebook
- Analyzed data from RAPID runs in order to find average CTs for each specimen if needed and LOD for runs using the standard curves.
- Incorporated lab data into abstract
- Initiated methods paper, format, data to include.

Wednesday 30 0800-1700 (1 hour lunch)

Conduct research for abstract, Pre-proposal preparation and completion of IRB essay.

Results review. Continue research for abstract. Finalize proposal. Finalize essay on IRB. Continue writing abstracts. Symposium planning.

1300-1700

Results review. Abstract preparation and pre-proposal/SOW development.

- Finalized abstract for submission under ASCP format. Verified data and double-checked abstract with proofreads by participating staff/researchers
- Continued working on paper outlining methods of experiment.
- Submitted abstract to ASCP electronically (before 30 May deadline).

Thursday 31

0800-1200

Results review, abstract preparation, and complete proposal development exercise.

Organize and archive research results and symposium planning.

Continued working on paper outlining methods of experiment.

1200 -1700 Pack and prepare for departure (hotel check-out).

Friday 1

- Transportation to airport, Depart Bangkok (~ 0500) / Arrive U.S. (~ 2000)

NOTE: GME students will be responsible for completing country clearance and readiness requirements and making travel arrangements (air and lodging) with support from Resident Office and investigators.

GME students will be responsible for funding required for travel to present results.

Appendix B

Pathology Resident Research Electives (from Dept. of Pathology Handbook)

Research Elective 144

Research Elective Rotation

Course Director:

All activities will be supervised by;

Director of Resident Research: Dr. Wade Aldous

Residency Program Director and/or Associate Director: Drs. Daniel Cruser and Dale Selby.

Resident Research Mentor: Dr. Peter Pelletier

Rotation period: Elective rotation, offered for 1 month.

General organization: Participation in research during residency training
Can provide valuable experience regardless of ultimate career goals and is a SAUSHEC graduation requirement.

As such, the Department of Pathology offers a Research Elective to provide protected time for participation in a research project, as well as support in all phases of conception and implementation of projects. Using elective time is not required for completion of the graduation research requirement, however, and residents may choose to do research without taking this elective.

Pathology residents may receive elective credit for up to 4 months of research time, which need not be contiguous, during their PGY-2 thru PGY-4 years. It is anticipated that most research projects will take place over the course of several months to four years, with protected elective time allocated for periods of intensive work such as background literature reviews, data collection, or data analysis.

Rotation Goals and Objectives: The goal of the resident research program is for the resident physician to gain a better understanding of the scientific method and the acquisition of new knowledge through a mentored research experience. The resident will demonstrate ACGME competencies in medical knowledge, practice-based learning and improvement, interpersonal and communication skills, and professionalism.

Reference: AGGME

General objectives of the Pathology Research Elective are to:

1. Acquaint the resident with a particular area of pathology-related research.

2. Teach the resident appropriate research techniques and research design.

3. Assist the resident to complete and write up for publication the results of their research
Specific resident learning objectives for the research project are:

To learn to develop a research question (Medical Knowledge and Practice-Based Learning).

To learn to access, critique, and assimilate the current medical literature pertaining to the research topic (Practice-Based Learning).

To gain an understanding of the scientific method by learning to write an IRB approved research protocol (Practice-Based Learning).

To learn and understand the purpose of informed consent and the regulatory approval process in the setting of research ethics by completing the HIPPA compliance training and obtaining IRB approval for the proposed research project (Professionalism and Systems-Based Practice).

To perform the research and develop the necessary skills required to do this such as laboratory techniques and computer skills (Practice-Based Learning).

To learn and apply the appropriate data analysis and basic biostatistics needed for the project (Practice-Based Learning).

Research Elective 145

To demonstrate communication skills by presenting research results to program directors and fellow residents and/or presenting results at a national meeting and/or writing a paper for publication in medical journals (Interpersonal and Communication Skills).

Resident Duties and Responsibilities:

To receive elective credit for research, the resident must complete the following minimum requirements:

. Identify a faculty research mentor and proposed project.

. Submit a brief (1-2 page) summary of a proposed research project and a research plan with study design and timeline (which may consist of the IRB protocol) to be approved by Residency Program Director or Associate Director and the research mentor.

. Complete the Collaborative Institutional Training Initiative (CITI training) on line research training module.

- . Obtain regulatory approval for the project, as appropriate. In most cases this will include writing and submitting a protocol to the IRB.
- . Present findings to fellow residents and program directors or at a national meeting in the form of a poster or as a publication in a medical journal.
- . Submit a final product to the program directors. This may be an abstract, a poster presentation, the draft of a paper, or a publication.
- . Attend all regularly-scheduled academic conferences, other military duties, and conferences as assigned.
- . Obtain prior approval for time spent away from the primary training sites (BAMC and WHMC).

Outcomes assessment: Subjective - A standard competency-based trainee evaluation will be completed at the end of the rotation by the faculty research mentor.

Additional Information

Note that use of the term "research" may be interpreted broadly to encompass a range of scholarly pursuits. Dr. Aldous and the program directors are available to help residents identify potential research mentors and scholarly projects. Residents also have access through the medical center to many resources ranging from computer classes, seminars on clinical investigation, and statistics help.

The requirements listed above are only minimal requirements. It is hoped that participating residents will also take advantage of the research elective opportunity to develop new skills, present at national meetings, and write up the results of their research for journal publication.

SAUSHEC Graduation Paper Requirement

Research Opportunities

1. Several pathology staff have ongoing projects in which you can participate or start. These are usually presented at the Research Committee Meeting.
2. Cancer Therapy and Research Center (CTRC) collaboration. The CTRC has many opportunities for residents to participate in original research. Most projects involve benchtop work using molecular techniques. These projects are designed to result in a publication.
3. Elective research month. You can use an elective month or more for research. See the handbook for details.

Timeline

1. You should have a project by the end of your second year.
2. Plan to submit your manuscript by the middle of your senior year, at the very latest.
3. Warning!!!! The SAUSHEC Graduate Medical Education Committee (GMEC) starts to review resident compliance with the SAUSHEC research requirement by early Fall of your senior year. Program Directors are required to present non-compliant residents by name to the GMEC. Continued non-compliance will result in adverse action. It is SAUSHEC policy that if you do not complete the research requirement you will not receive your graduation certificate.

Evaluation

All projects are graded by the Program Director or Associate Program Director using the standard score sheet on the SAUSHEC web site (see below). A minimum score of 60 is required in order to graduate. In general, if you are first author on a publication in any of the categories listed above, you will likely pass.

SAUSHEC Graduation Paper Requirement

SAUSHEC GRADUATION PAPER

SCORE SHEET

Total Percentage Points: 100%

A score under 60% is considered unsatisfactory.

1. Originality of project (10 pts)

Score

1 2 3 4 5 6 7 8 9 10 _____

Comments:

2. Review/Discussion of Literature /Quality of Introduction (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

3. Design of Clinical or Animal Research/Case Report/Education project/ Chart or Subject Review (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

4. Data Analysis/Results/Graphics (20 pts)

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20 _____

Comments:

5. Quality of Discussion (20 pts)

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20 _____

Comments:

6. Effort required to design and execute Study/Project (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

7. Scientific/Academic merit/significance of project (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

8. Style (Sentence structure/grammar/clarity of thought) (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

TOTAL = _____

PROGRAM DIRECTOR: _____

Signature Date

Appendix C

Through earlier AF/SGR funded projects we developed a highly sensitive and specific, dual-fluorogenic, hydrolysis probe (TaqMan), RAPID/JBAIDS PCR assays for the detection of *Cryptosporidium* species.

The *Cryptosporidium* assay limit of detection (LOD) was established at ≤ 1000 fg (≤ 100 genomic equivalent) (Table C1 and Figure C1). Assay *in vitro* sensitivity was 100% and specificity 100% concordant with well characterized genetic near neighbors and clinically significant organisms (Table C2 and Figure C2). Assay *in vitro* sensitivity conducted with 12 *Cryptosporidium* positive stool samples was 100% concordant with culture results (Figure C3).

Cryptosporidium wet assay tests results follow:

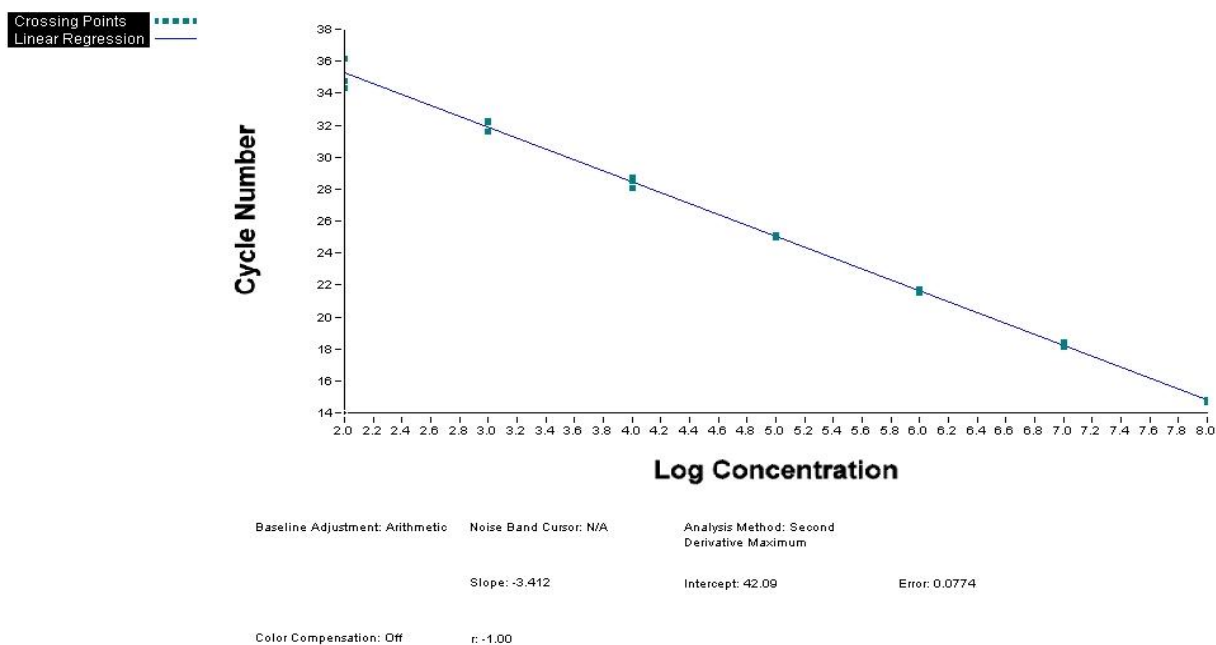
The reaction contained: 1x of 4.0 mM MgCl₂ buffer, 1x of dNTP buffer, 1x of stab buffer, 0.3 μ mol of each primer, 150 nmol TaqMan probe, 0.32 μ L of Taq:AB, 1.68 μ L of enzyme diluent and 2 μ L of sample lysate in a total volume of 20 μ L. The reaction containing TaqMan probe and primers was incubated and fluorescent signal detected simultaneously on R.A.P.I.D. platform. The thermocycling parameters were heat-activated at 95°C for 3 min followed by 45 cycles consisting of denaturation at 95°C for 0 seconds, annealing and polymerization steps were combined at 60°C for 20 sec.

Cryptosporidium Assay Detection Limit

Triplicate reactions for each 10-fold dilution of cloned target fragment were analyzed to establish a standard curve.

Table C1. Standard curve of *Cryptosporidium* assay

Sample Name	Copies/reaction	C _T average
P_CR D2	1.38E+08	14.71
P_CR D3	1.38E+07	18.23
P_CR D4	1.38E+06	21.64
P_CR D5	1.38E+05	25.06
P_CR D6	1.38E+04	28.45
P_CR D7	1.38E+03	32.02
P_CR D8	1.38E+02	35.09



Note: R^2 of unity is based on the robustness of linearity achieved by the PCR. For optimized reactions that have met validation criteria, a “Best Fit” algorithm is utilized to automatically calculate correlation at an assumed value of unity.

Table C2. Strains and number of enteric pathogens used for cross-reactivity test.

Pathogen	No. of sample	PCR Interpretation
<i>Acinetobacter calcoaceticus</i>	1	Negative
<i>Arcobacter butzleri</i>	1	Negative
<i>Campylobacter spp.</i>	7	Negative
<i>Citrobacter freundii</i>	1	Negative
EHEC	1	Negative
<i>Enterobacter aerogenes</i>	1	Negative
<i>Enterobacter cloacae</i>	1	Negative
Enterotoxigenic <i>E. coli</i> (ETEC)	2	Negative
Enteroinvasive <i>E. coli</i> (EIEC)	1	Negative
<i>Escherichia coli</i>	1	Negative
<i>K. pneumoniae</i>	1	Negative
<i>P. aeruginosa</i>	1	Negative
<i>Proteus hauseri</i>	1	Negative
<i>Salmonella spp.</i>	2	Negative
<i>Staphylococcus spp.</i>	4	Negative
<i>Vibrio spp.</i>	2	Negative
Total	28	

Figure C2. Amplification curves obtained on R.A.P.I.D. platform tested against enteric bacteria

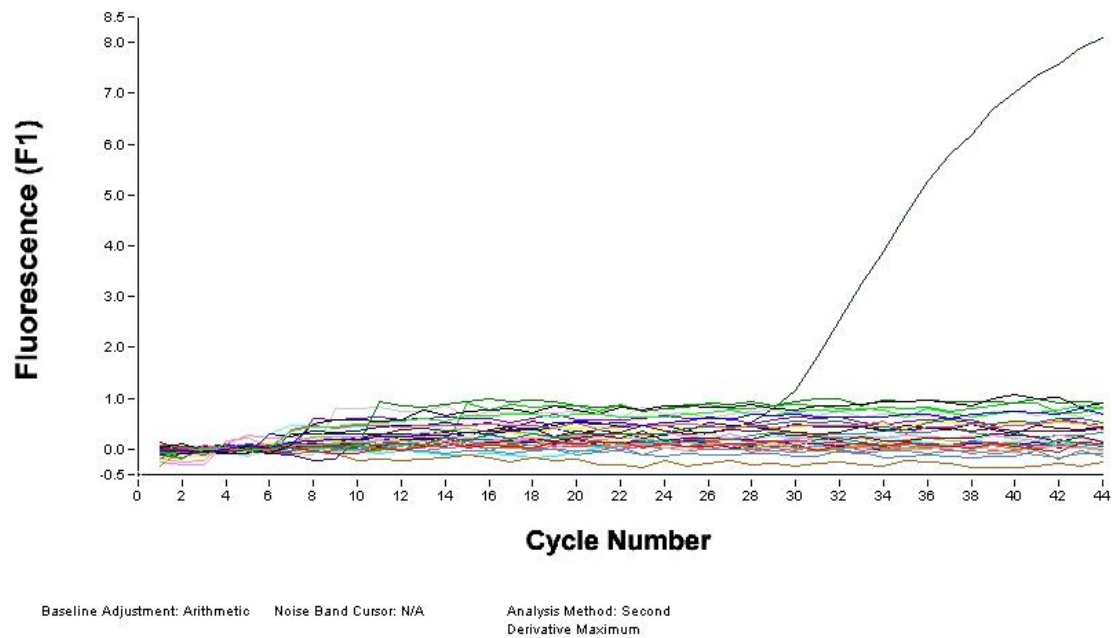


Figure C3. Amplification curves obtained on R.A.P.I.D. platform tested against 12 positive stool samples (including 2 positives and 2 negatives)

